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Dog nectin-4 is an epithelial cell receptor for canine distemper virus that facilitates virus entry and syncytia formation

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ABSTRACT

Canine distemper virus (CDV) was shown to use dog nectin-4 as a receptor to gain entry into epithelial cells. RNA from dog placenta or MDCK kidney cells was isolated and cDNAs were prepared. Two splice variants of dog nectin-4 were identified. A deletion of 25 amino acids was found in the cytoplasmic domain of dog nectin-4 from MDCK cells, corresponding to a splice variant that is also seen in murine nectin-4, and did not affect its role as a receptor. Both dog nectin-4 and human nectin-4 could function as an entry factor for CDV containing an EGFP reporter gene. Inhibition of dog nectin-4 expression by RNAi or nectin-4 antibodies decreased CDV titers and EGFP fluorescence. Finally, dog nectin-4 also promotes syncytia formation, which could be inhibited by siRNA treatment. These data confirm that dog nectin-4 can be used by CDV to gain entry into epithelial cells, and facilitate virus spread.

Introduction

The family *Paramyxoviridae*, within the order *Mononegavirales*, consists of four genera, *Morbillivirus*, *Paramyxovirus*, *Rubulavirus* and *Pneumovirus*. Morbilliviruses are highly contagious pathogens that are responsible for some of the most devastating diseases that affect mammals worldwide. Members of this genus include measles virus (MeV), canine distemper virus (CDV), peste des petits ruminants virus (PPRV), rinderpest virus (RPV), phocine distemper virus (PDV) and dolphin morbillivirus (DMV) (Diallo, 1990; Rima and Duprex, 2006).

Canine distemper virus (CDV) produces a contagious disease that elicits high mortality in a wide range of terrestrial carnivores (Appel, 1970; Barrett, 1999; Deem et al., 2000; Harder and Osterhaus, 1997; Martella et al., 2008). CDV is closely related to MeV in terms of gene organization and genome sequences (Harder and Osterhaus 1997; Barrett 1999). Like MeV, CDV is a negative strand RNA virus whose RNA genome is associated with nucleocapsid protein (NP), phosphoprotein (*P*), and an RNA polymerase (*L*). Surrounding the nucleocapsid is a membrane envelope that contains 2 glycoproteins, the hemagglutinin (*H*), used for attachment to the host cell receptor, and the fusion (*F*) protein, required for entry and syncytia formation. A matrix protein (*M*) lies on the inner side of the viral envelope and helps maintain the integrity of the virus. Both MeV and CDV produce similar symptoms and have comparable incubation periods in their respective natural hosts. However, CDV has a broader host range than MeV, and can infect dogs, coyotes, wolves, cats, lions, tigers, minks, ferrets, raccoons, pandas, bears, and civets (Martella et al., 2008). CDV exhibits a higher frequency of neurological involvement than MeV, which can vary with different strains of the virus (Beineke et al., 2009). Ferrets are particularly susceptible to neurotropic strains of CDV and have proven to be an excellent small laboratory animal model for studying the spread of virus from the lymphatic system, to epithelial cells, and subsequently the central nervous system (Ludlow et al., 2012; Sawatsky et al., 2012; von Messling et al., 2003) The virus enters the canine host by the nasal or oral route and initiates replication in lymphoid tissues. In dogs, the incubation period may range from 1 to 4 weeks. Transient fever peaks 3 to 6 days after infection as the virus spreads throughout the lymphatic system. At 6 to 9 days post-infection, CDV spreads to epithelial cells in most organs. Respiratory, intestinal, and dermatologic symptoms are evident 10 days post-infection. The infected animal exhibits loss of appetite, ocular and nasal discharge, and tonsillitis. Like MeV, CDV infection is associated with transient immunosuppression

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that may promote morbidity and mortality (Iwatsuki et al., 1995; Krakowka et al., 1980; von Messling et al., 2004). If the host immune response is weak, the virus replicates extensively in the epithelial cells and subsequently the central nervous system. Demyelinating encephalitis can occur when the virus persists and establishes chronic infections in the brain (Beineke et al., 2009). These can produce neurological symptoms between 20 and 50 days post-infection. In the pre-vaccine era, canine distemper disease was endemic in domestic dogs (Martella et al., 2008, 2007). From the 1960s onward, attenuated canine distemper virus vaccines contributed to a decrease in the percentage of CDV-infected domestic dogs in countries with high vaccine coverage, leaving wild carnivores as the main reservoir (Croghan, 1966; Keeble, 1962; Rockborn, 1959).

Both CDV and MeV enter susceptible cells using surface protein receptors. Three cellular receptors have been identified to date. The signaling lymphocyte activation molecule (SLAM), which is expressed on the surfaces of activated T- and B-lymphocytes, macrophages, and dendritic cells (Wang et al., 2004), serves as an immune cell receptor for morbilliviruses (Baron, 2005; Hsu et al., 2001; Seki et al., 2003; Tatsuo et al., 2000, 2001). CD46, an inhibitor of complement activation, acts as a cellular receptor for vaccine and laboratory adapted strains of measles viruses (Dorig et al., 1993; Naniche et al., 1993). Recently, nectin-4 (poliovirus-receptor-like-4, PVRL4) has been identified as an epithelial cell receptor for MeV and CDV (Muhlebach et al., 2011; Noyce et al., 2011; Noyce and Richardson, 2012; Pratakpiriya et al., 2012). The V domain of nectin-4 was shown to bind strongly to MeV hemaglutinin (H) protein (Muhlebach et al., 2011). A recombinant CDV, containing mutations in the H protein that make it blind to the epithelial receptor, infects the lymphatic system to produce immunosuppression, but cannot spread to epithelial cells to produce clinical disease and virus shedding (Sawatsky et al., 2012). Nectin-4 was also recently suggested to be an epithelial and neurotropic receptor for CDV but the sequence of dog nectin-4 and a comprehensive characterization of this receptor was not reported (Pratakpiriya et al., 2012).

The nectin family belongs to the immunoglobulin (Ig) superfamily comprised of at least five members, including nectins 1–4 and the prototypic poliovirus receptor (PVR) (Takai et al., 2008). They consist of three Ig-like ectodomains (V, and two C2 domains), in addition to a transmembrane region, and a cytoplasmic tail. V domains are involved in homotypic or heterotypic interactions between the nectins, whereas C2 domains enhance the affinity of these interactions (Fabre et al., 2002; Satoh-Horikawa et al., 2000; Takai et al., 2008). Nectins are normally localized to the adherens junctions, where they define cell-to-cell interactions and boundaries (Kurita et al., 2011; Takai et al., 2008). Interestingly nectin-4 expression is up regulated on the surface of many different types of cancer cells and has been suggested to be a tumor cell marker for lung, ovarian and metastatic breast cancers (Derycke et al., 2010; Fabre-Lafay et al., 2005; Fabre-Lafay et al., 2007; Takano et al., 2009). This observation could support the use of MeV as an oncolytic agent for treating a variety of cancers (McDonald et al., 2006; Russell and Peng, 2009).

In this study we confirmed that CDV uses dog nectin-4 as a receptor to gain entry into host cells. Two variants of dog nectin-4 were identified from either dog placenta or MDCK tissue culture cells. The major difference between these two proteins was the deletion of 25 amino acids in the cytoplasmic domain of one of the variants. However, this deletion did not yield any difference in the ability of CDV to infect cells expressing these dog nectin-4 proteins. Overexpression of dog nectin-4 in Vero monkey kidney cells resulted in localization of the protein to the cell surface, similar to what was observed previously with human nectin-4. Both dog nectin-4 and human nectin-4 could function as an entry factor for CDV that contained an enhanced green fluorescent protein (EGFP) reporter gene. Inhibition of dog nectin-4 expression by RNAi, or blocking virus–receptor interaction with polyclonal antibodies directed against nectin-4, resulted in decreased CDV titers and EGFP fluorescence. Finally, dog nectin-4 also promotes efficient viral spread and syncytia formation, which could also be inhibited by siRNA treatment. Taken together, these data confirm that dog nectin-4 can be used by CDV to gain entry into epithelial cells, and spread from cell to cell.

Results

Wild-type CDV strain 5804PeH infects cells expressing human or dog nectin-4

CDV and MeV are both members of the *Morbillivirus* genus in the family *Paramyxoviridae*. Previous findings have shown that members of this genus share common tropism and pathologies, including the use of SLAM as a cell receptor on cells of the immune system (Baron, 2005; Hsu et al., 2001; Seki et al., 2003; Tatsuo et al., 2000). We first investigated whether wild-type CDV strain 5804PeH could use human nectin-4 as a receptor to gain entry and mount a productive infection in these cells. Vero cells overexpressing human nectin-4 were very susceptible to CDV 5804PeH, producing large GFP positive syncytia compared to control Vero cells (Fig. 1). The human breast cancer cell line, MDA-MB-231, previously shown to be negative for nectin-4 expression and MeV susceptibility (Noyce et al., 2011), was only susceptible to CDV 5804PeH infection if human nectin-4 was expressed in these cells (Fig. 1; compare MDA-MB-231 and MDA-MB-231.hPVRL4). The human nectin-4 positive breast cancer cell line MCF-7 was also susceptible to wild-type CDV infection, suggesting that wild-type CDV 5804PeH is able to use the human nectin-4 receptor to gain entry into cells.

We next wanted to confirm that CDV 5804PeH could infect a more relevant dog epithelial cell line, MDCK. We obtained a number of MDCK cell lines and determined whether they were susceptible to wild-type CDV infection (Fig. 1; MDCK-super tubes, MDCK-super domes, and MDCK). We observed that only MDCK variants expressing dog Nectin-4 were susceptible to CDV infection. Interestingly, MDCK-super domes had a lower level of nectin-4 surface expression and a small number of syncytia following infection with CDV 5804 compared to MDCK cells, suggesting that the abundance of nectin-4 on the cell surface dictates its infectivity by wild type strains of CDV. The MDCK-super tube cell line did not express nectin-4 and was not susceptible to infection by CDV. Overall, these data suggest that wild-type CDV can use both human and dog homologues of nectin-4 to gain entry into cells *in vitro*.

Molecular cloning of the cDNA for dog nectin-4

We investigated whether wild type strains of CDV could use the dog homologue of nectin-4 as a cellular receptor. To this end, we isolated two putative clones of dog nectin-4 from either Labrador retriever (LR) placenta or MDCK tissue culture cells (Fig. 2A). These two clones differed in that the MDCK nectin-4 clone had a 25 amino acid deletion in the endodomain (aa 412–436) as a result of alternative splicing (Fig. 2B). This splice variant aligned seamlessly with the mouse variant 2 at this region. Thus, similar to murine nectin-4, the dog nectin-4 gene encodes at least two splice variants that can both act as receptors for wild-type CDV. Interestingly, the dog nectin-4 sequences obtained were different from the dog nectin-4 sequence predicted from the female boxer dog genome

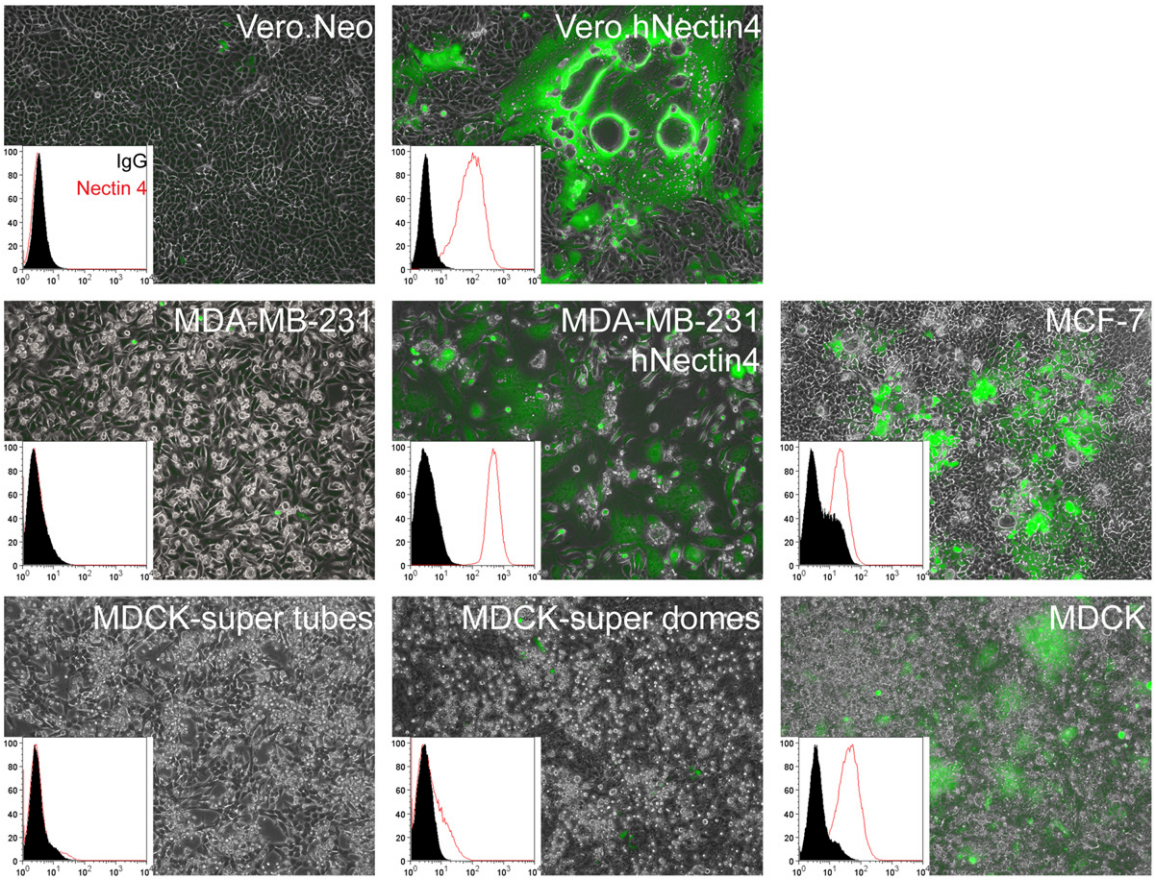

Fig. 1. Wild-type CDV 5804PeH can use either human or dog nectin-4 to gain entry into cells. Cells of monkey (Vero or Vero.hPVRL4; MOI 0.5), human (MDA-MB-231, MDA-MB-231.hPVRL4, MCF-7; MOI 5.0), and dog (MDCK-super tubes, MDCK-super domes, MDCK; MOI 5.0) origin were used to infect with CDV 5804PeH at the indicated MOI. Fluorescence images were captured four days post infection and overlaid with phase contrast images. The inset histograms depict the cell surface expression profile of nectin-4 in the corresponding cell line (isotype ctrl antibody, IgG – shaded; nectin-4 antibody, nectin-4 – red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(NCBI Genbank accession XM847277.2); the predicted boxer sequence was truncated by the insertion of a stop codon at amino acid 438. This may be another variant but could also be the result of a genome sequencing error. Overall, the full-length LR and shorter MDCK nectin-4 sequences were 94.3% and 89.9% identical at the amino acid level to human nectin-4, respectively. Identical full-length and short nectin-4 sequences were determined from cDNAs prepared from the placenta of a papillon dog (NCBI Genbank KC165685 and KC165686). The V domain of nectin-4 has been implicated in receptor binding (Muhlebach et al., 2011), and the dog homologue contains only 3 changes in this region, A31P, P92A, and N106S, compared to its human counterpart (Fig. 2A).

Cell lines transduced with retroviruses containing the dog nectin-4 gene express the receptor on their cell surface

Although the dog nectin-4 cDNA clones were highly similar to human nectin-4 at the amino acid level, we wanted to confirm that these glycoproteins localized to the cell surface. The expression pattern of these cDNAs in Vero cells was analyzed by immunofluorescence microscopy (Fig. 3). To generate cell surface expression of these molecules, we constructed retrovirus expression vectors expressing the membrane-bound form of human (Vero.hNectin-4), MDCK (Vero.MDCKNectin-4), and Labrador retriever nectin-4 (Vero.LRNectin-4). Vero cells were transduced with VSV-G pseudotyped retrovirus particles created in the Phoenix amphotropic cell system (Swift et al., 2001). Stable cell lines expressing the nectin-4 proteins were selected in the presence of G418, followed by immunofluorescence staining and confocal microscopy (Fig. 3A) or flow cytometry (Fig. 3B). Both the MDCK nectin-4 and LR nectin-4 proteins were localized to the plasma membrane according to confocal microscopy analysis, as was human nectin-4. Flow cytometry confirmed that the majority of cells in the stable population expressed nectin-4. Human nectin-4, MDCK nectin-4, and LR nectin-4 cell lines exhibited increased fluorescent signals for nectin-4 compared to the Vero.Neo negative control cells.

CDV replicates efficiently in cells that express dog nectin-4

To determine whether dog nectin-4 could be used as a receptor for the CDV wild-type strain 5804PeH, the Vero stable cell lines expressing MDCK or LR nectin-4 were infected at an MOI of 0.5 and syncytia formation and virus titers were monitored over a 3d time course (Fig. 4). Both clones of dog nectin-4 induced formation of syncytia of comparable sizes following infection with CDV at 3d post-infection (Fig. 4A). Interestingly, syncytia formation in the nectin-4-expressing cells seemed to be larger compared to those in the VerodogSLAMtag cells. Vero.Neo control cells exhibited a low background infection with CDV 5804PeH, as evidenced by isolated single fluorescent cells which did not produce syncytia. Similar observations were previously made with MeV infections of Vero cells (Hashimoto et al., 2002). By day 3-post infection, CDV 5804PeH virus titers were higher in the Vero cells expressing dog SLAM, MDCK nectin-4, or LR nectin-4, as compared to the Vero.Neo control cells (Fig. 4B). The CDV 5804PeH

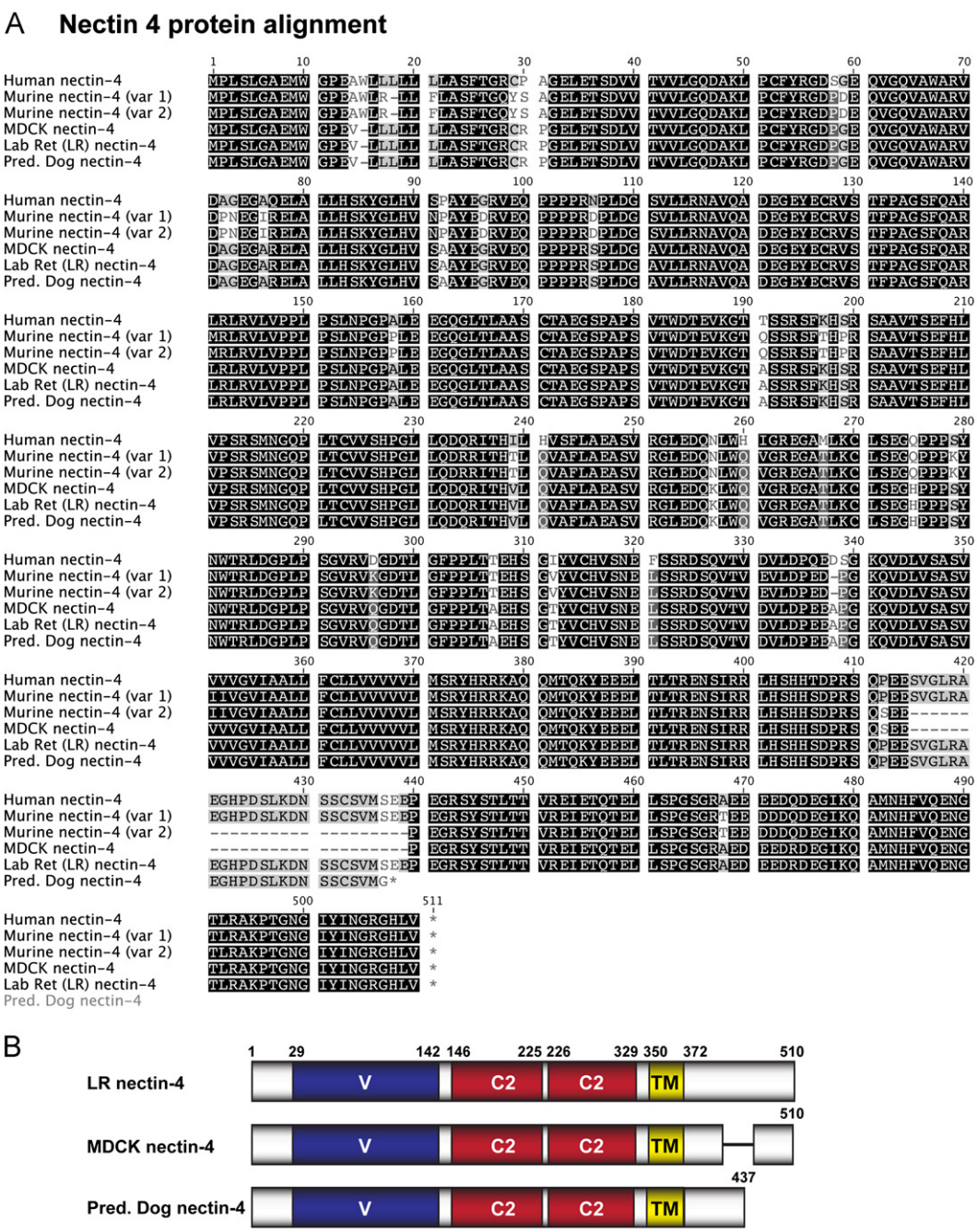

Fig. 2. Amino acid sequences of human, mouse and dog (MDCK cell and Labrador retriever) nectin-4. (A) The amino acid sequences of human, mouse variant 1 (var 1) and variant 2 (var 2), Labrador retriever (LR), and MDCK nectin-4 are aligned. Residues having similarity are shaded (dark shading represents identical residues; light shading represents conservative changes). (B) Schematic diagrams of the two dog nectin-4 clones from Labrador retriever placenta (LR Nectin-4) or MDCK cell culture cells (MDCK Nectin-4) compared to the predicted dog nectin-4 (Pred. Dog nectin-4). The Ig V and C2 domains, along with the transmembrane domain (TM) are indicated.

virus replicated to similar titers in Vero.dogSLAM, Vero.MDCKNectin-4 and Vero.LRNectin-4 cell lines (Fig. 4B). hNectin-4 also appeared to function equally well as a receptor for CDV (Fig. 1).

The affinity of MeV H protein for Nectin-4 receptor was previously reported to be 4–5 times greater than for SLAM/CD150 (Muhlebach et al., 2011). This could potentially result in increased efficiency of infection by CDV or measles virus when using nectin-4 as a receptor. This did not appear to be the case with CDV 5804PeH virus above, nor was it true when a stable Vero cell line expressing human nectin-4 (hNectin-4) was infected with wtMeV (IC323-EGFP) at an MOI of 0.05 (Fig. 5). Significant syncytia formation in both the hSLAM and hNectin-4 cell lines was observed by 32 h post infection (Fig. 5A). Just as was the case with CDV, MeV replicated to similar levels in hNectin-4 expressing cells compared to those observed in hSLAM-expressing cells (Fig. 5B). Collectively, these data suggest that wild-type CDV 5804PeH and IC323 wtMV-EGFP can use nectin-4 and SLAM equally well as cellular receptors for virus entry and pathogenesis.

Diminished dog nectin-4 expression or inhibition by polyclonal antibodies yields inefficient CDV infection

To confirm that dog nectin-4 was a *bona fide* receptor for wild-type strains of CDV, siRNA or antibody inhibition assays were utilized in attempts to block the initial stage of virus attachment

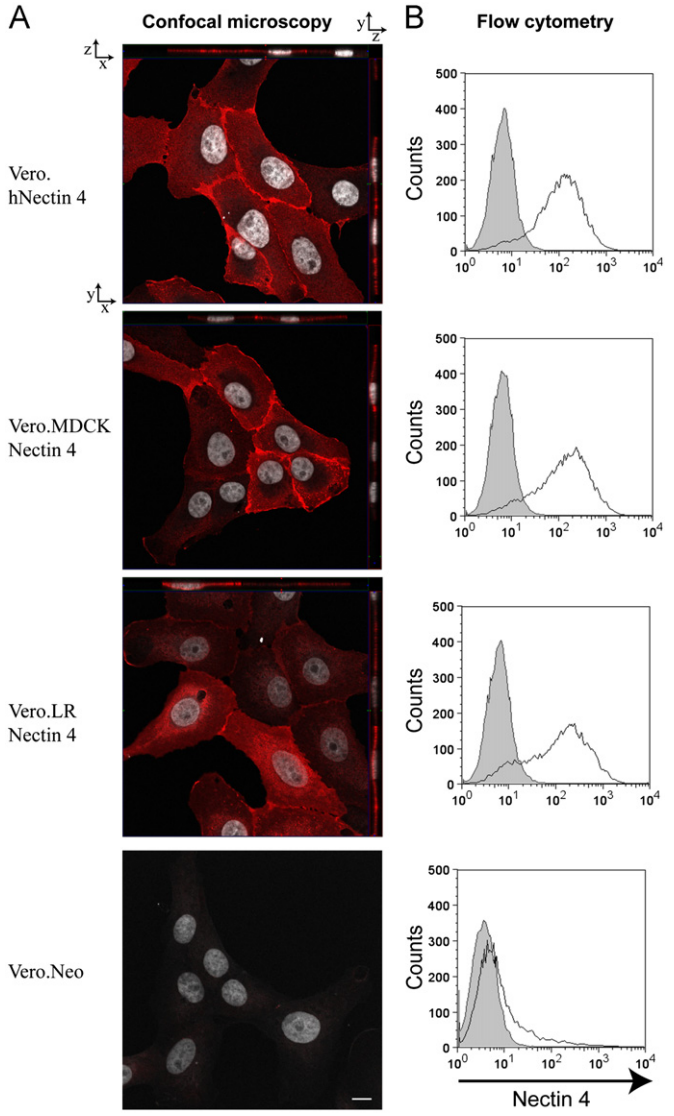

Fig. 3. Dog nectin-4 is localized to the cell surface. (A) Vero stable cell lines expressing human (Vero.hNectin-4), MDCK (Vero.MDCKNectin-4), or LR (Vero.LR-Nectin-4) nectin-4 were grown on glass coverslips, fixed in paraformaldehyde, and stained with a goat anti-human nectin-4 polyclonal antibody (red). Nuclei were visualized with TO-PRO-3 nuclear stain (grey). Three hundred nanometer image z-stacks were captured on a Zeiss upright confocal microscope and analyzed using ZEN 2011 image capture software (Zeiss). Scale bar = 10 μm. (B) Flow cytometry analysis reveals dog nectin-4 is expressed on the cell surface of Vero MDCK nectin-4 and LR nectin-4 cells. Vero stable cell lines were incubated with phycoerythrin (PE)-conjugated mouse monoclonal antibody specific for human nectin-4, or a PE-conjugated mouse IgG2a control antibody (shaded histogram). The *Y*-axis represents cell counts and the *X*-axis represents fluorescence intensity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

prior to infection. siRNA against MDCK nectin-4 was used to knock down exogenous levels of MDCK nectin-4 in the Vero stable cell line. A scrambled control, individual MDCK nectin-4 siRNAs (siRNA 1 or siRNA 5), or a pool of 4 siRNAs that were specific for either MDCK nectin-4 (siRNA pool) were transfected into Vero.MDCKNectin-4 cells for 72 h. Analysis by flow cytometry demonstrated that dog nectin-4 surface expression was reduced by 75–80 % in the MDCK nectin-4 siRNA-treated samples, compared to control siRNA-treated cells (Fig. 6A, lower panel). The cells were subsequently infected with CDV 5804PeH, after which syncytia formation and virus titers were monitored 3d post infection (Fig. 6B and C). The number and size of syncytia were dramatically decreased in the MDCK nectin-4 siRNA treated cells (Fig. 6B) compared to the control siRNA and mock treated samples. CDV titers decreased approximately 2-logs in the MDCK nectin-4 siRNA treated cells, indicating that CDV uses dog nectin-4 to infect these cells. Interestingly, RNAi treatment with siRNA 1 was just as effective at inhibiting the surface expression of nectin-4 and preventing the infection by CDV as the siRNA pool.

These data were confirmed using nectin-4 polyclonal antibodies to block the attachment of CDV to the dog nectin-4 receptor. A rabbit polyclonal antibody directed against human nectin-4 was used to inhibit binding of CDV to cells expressing MDCK nectin-4. Vero.MDCKNectin-4 cells were pre-treated with varying concentrations of human nectin-4 antibodies, followed by infection with CDV 5804PeH at an MOI of 0.5. After the inoculation period, cells were washed rigorously to remove any unbound virus, and subsequently incubated with media. Incubation with polyclonal antibodies directed against human nectin-4 effectively blocked the number and size of syncytia, compared to the IgG control-treated cells (Fig. 6D). We also observed an approximate 2-log decrease in the titer of CDV produced from cells treated with nectin-4 antibodies, compared to the IgG controls (Fig. 6E). Taken together, the siRNA and nectin-4 antibody inhibition studies strongly indicate that wild-type strains of CDV can use dog nectin-4 as a cellular entry receptor.

Efficient CDV cell to cell spread requires dog nectin-4

We next investigated whether nectin-4 was also involved in cell to cell spread of CDV in cultured cells. VerodogSLAMtag cells were infected with wild-type CDV for 90 min at an MOI of 4, trypsinized and mixed with uninfected Vero.MDCKNectin-4 cells that had been treated with control or MDCK nectin-4 siRNA (ratio 1:5, infected:uninfected siRNA treated cells). Following attachment of the cells to the culture plate, they were overlaid with medium containing methylcellulose, and syncytia were counted 3d later. We observed a marked reduction in the number and size of infected foci in the Vero.MDCKNectin-4 cells that had been treated with MDCK nectin-4 siRNA, compared to mock or control siRNA-treated cells (Fig. 7, compare panel A and B with panel C). Overall, these data suggest that nectin-4 is responsible for both initial virus entry, and cell-to-cell spread between cells expressing dog nectin-4.

Discussion

Our laboratory and another have previously demonstrated that MeV attachment and entry into primary human epithelial cells and adenocarcinoma cell lines require the epithelial cell receptor, nectin-4 (Muhlebach et al., 2011; Noyce et al., 2011; Noyce and Richardson, 2012). In the current study we show that dog nectin-4 serves as an epithelial receptor for CDV, another related member of the *Morbillivirus* subgroup of family *Paramyxoviridae*. Wild-type CDV 5804PeH could use both human and dog nectin-4 as a receptor on cells. Specific knockdown of dog nectin-4 reduced its overall surface expression, resulting in a significant decrease in CDV infectivity in Vero.MDCKNectin-4 and Vero.LRNectin-4 cells. Consistent with this finding, neutralizing antibodies directed against nectin-4 could also inhibit CDV infection of these cells. Finally, dog nectin-4 expression was found to contribute to efficient cell-to-cell spread of CDV.

During morbillivirus infections, aerosols containing the viruses enter the upper respiratory tract and target dendritic cells and macrophages via DC-SIGN or SLAM receptors (de Witte et al., 2008; Ferreira et al., 2010; Lemon et al., 2010; Muhlebach et al., 2011; Noyce et al., 2011; Noyce and Richardson, 2012). These

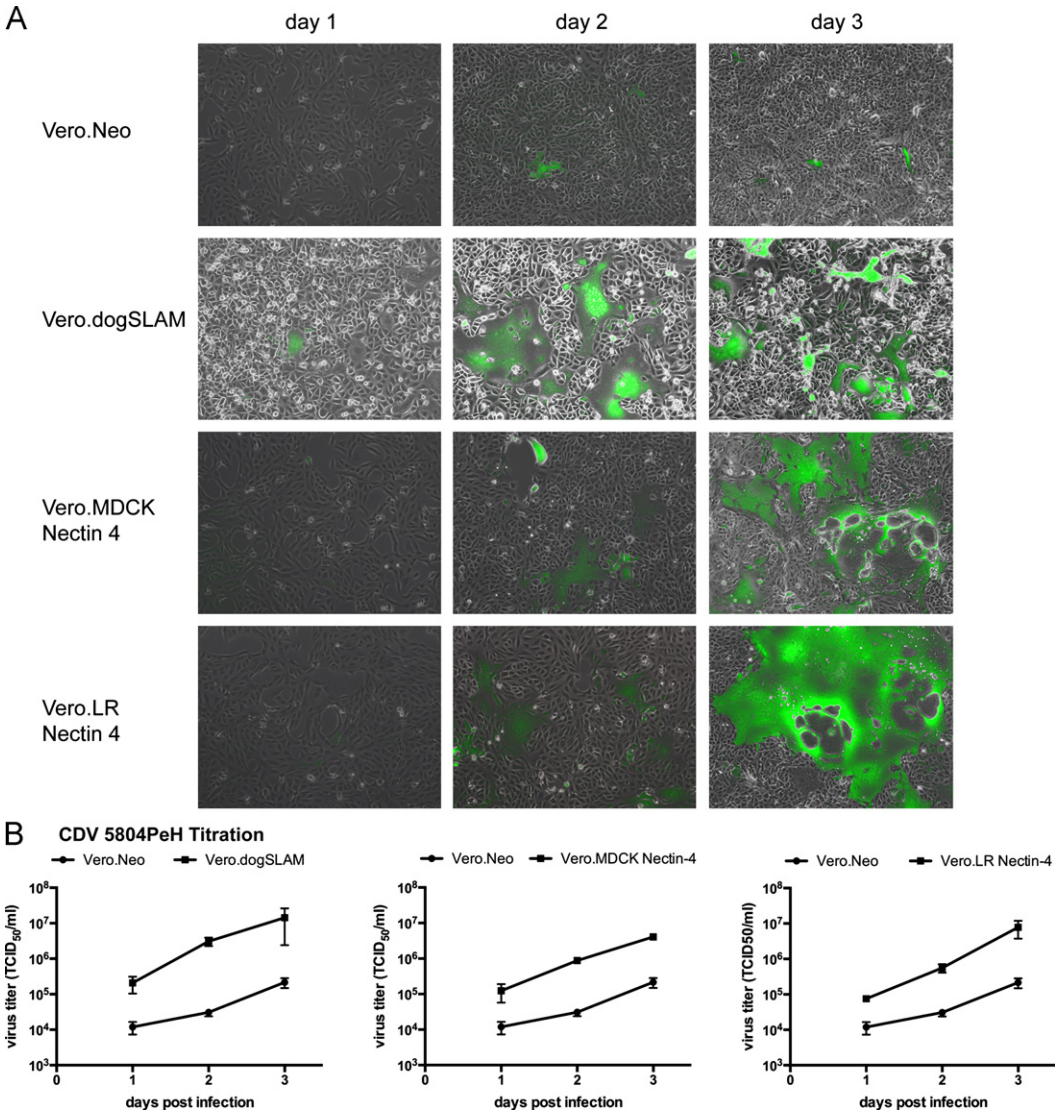

Fig. 4. Wild-type CDV 5804PeH efficiently infects dog nectin-4 expressing Vero cells. Vero stable cell lines expressing dog SLAM (Vero.dogSLAMtag), MDCK nectin-4, (Vero.MDCK Nectin-4), LR nectin-4 (Vero.LR Nectin-4), or a control plasmid (Neo) were infected at an MOI of 0.5 with CDV 5804PeH. (A) Phase contrast and fluorescence images were captured and overlaid to visualize the extent of virus replication (GFP) and syncytia formation over 3d. (B) Dog nectin-4 results in a significant increase in CDV 5804PeH virus titers compared to Vero cells alone. CDV-infected cells were harvested at various times post infection and titered on VerodogSLAMtag cells. Data are the means from three independent experiments, and error bars represent the SEM.

infected cells subsequently drain to the local lymph nodes, where viruses like MeV and CDV can infect a population of uninfected T and B cells expressing SLAM. Infected lymphocytes disseminate the virus throughout the host via the peripheral blood and lymphatic systems (Iwatsuki et al., 1995; Krakowka et al., 1980; Lemon et al., 2010; Sawatsky et al., 2012; von Messling et al., 2003, 2004). The virus disseminates to primary and secondary lymphoid organs as well as lymph nodes in the gastro-intestinal tract (Appel, 1970; Blixenkrone-Moller, 1989; Iwatsuki et al., 1995; von Messling et al., 2004). Finally, CDV enters the epithelial cells of the respiratory, gastro-intestinal, urinary, and endocrine systems via the epithelial receptor (Appel, 1970; Frisk et al., 1999; Ludlow et al., 2012; von Messling et al., 2004). This receptor functions as an exit receptor, promoting amplification and subsequent release of the virus from airway, urinary, and gastro-intestinal epithelial cells. All body secretions contain the virus and are very contagious. Recombinant CDV, containing a mutated H protein that could not recognize the epithelial receptor, infected immune cells and caused immunosuppression (Sawatsky et al., 2012). This mutated virus could not produce clinical signs of infection, spread to the central nervous system, nor was it shed from the infected host. Neurovirulent strains of CDV can subsequently infect cells of the central nervous system using receptors, which still remain uncharacterized (Ludlow et al., 2012).

MDCK cells are an epithelial cell line derived from the kidney of an adult female cocker spaniel. Over the years, numerous strains have been derived from the heterogeneous population of cells in the parental cell line, MDCK (NBL-2) (Dukes et al., 2011). These include the MDCK super tube and super dome cells used in Fig. 1. Parental MDCK cells have previously been used to isolate and propagate clinically relevant CDV strains (Dietzel et al., 2011; Lednicky et al., 2004; Mochizuki, 2006; Pillet and von Messling, 2009; Tan et al., 2011). In addition, MDCK cells failed to support the replication of CDV that contained H mutations making it blind to the epithelial cell receptor (Sawatsky et al., 2012). MDCK (NBL-2) cells can be infected with either wild type MeV or wild type CDV, and FACS analysis showed that these cells express nectin-4 on their

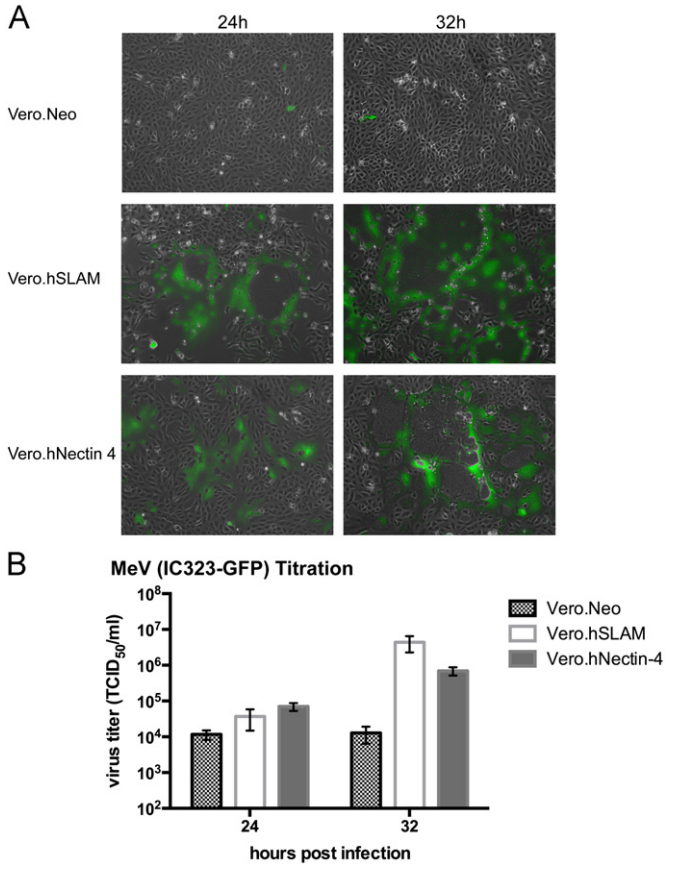

Fig. 5. MeV (IC323-GFP) replication of human nectin-4 expressing Vero stable cells. (A) Vero stable cell lines expressing human SLAM (Vero.hSLAM) or nectin-4 (Vero.hNectin-4) were infected at an MOI of 0.05 with wtMeV (IC323-GFP). Phase contrast and fluorescence images were captured and overlaid to visualize the extent of virus replication (GFP) and syncytia formation 32 h post infection. (B) Human nectin-4 expression results in an increase in wtMeV (IC323-GFP) compared to Vero cells alone (Neo). MeV-infected cells were harvested at 32 h post infection and titered on Vero-hSLAM cells. Data are means from three independent experiments, and error bars represent the SEM.

cell surface (Noyce et al., 2011). Based on this knowledge, we cloned and sequenced the nectin-4 epithelial receptor from these cells and provided evidence that dog nectin-4 can function as an epithelial cell receptor for CDV. Another variant of dog nectin-4 was identified from the placenta of a Labrador retriever, where it is highly expressed, which corresponds to the full-length protein that was previously reported (Reymond et al., 2001). These two clones differed in that the MDCK nectin-4 clone had a 25 amino acid deletion in the cytoplasmic endodomain (aa 412–436) as a result of alternative splicing (Fig. 2B). In addition, both splice variants were also isolated from the placenta of a papillon dog (GenBank KC165685 and KC165686), raising the possibility that at least two protein variants may be present within tissues that actively express dog nectin-4. The smaller dog nectin-4 variant aligned seamlessly with mouse variant 2 at this region. Both dog nectin-4 proteins can act equally well as receptors for wild-type CDV. Infections of Vero.MDCKNectin-4 and Vero.LRNectin-4 cell lines were more extensive than those in MDCK cells, due to their increased levels of nectin-4 expression. The titers of CDV produced from these cell lines were also much higher. Reduction of endogenous levels of dog nectin-4 with RNAi and inhibition of receptor–virus interactions with antibodies were less effective at preventing CDV infection in MDCK cells (data not shown). This is because MDCK cells are not as susceptible to CDV infection and serial passage of virus-infected cells is often required to increase the percentage of infected cells (Dietzel et al., 2011; Pillet and von Messling, 2009; Sawatsky et al., 2012). Second, MDCK cells exhibited very high doubling times, making the use of inhibitory antibodies and RNAi approaches less effective at preventing CDV infection. Consequently, Vero.MDCK-Nectin-4 and Vero.LRNectin-4 cells were chosen for the RNAi and nectin-4 antibody inhibition studies.

There is little species variation in nectin-4 sequences between human, mouse, and dogs ((Noyce et al., 2011); Fig. 2). Mouse nectin-4 can function as a receptor for MeV (Noyce et al., 2011) and human nectin-4 functions as a receptor for CDV (Fig. 1). Nectin-4 is an exit receptor that operates in the later stages of infection when virus is amplified and released from epithelial cells. Host tropism appears to be established during the initial stages of infection through use of the lymphocyte receptor SLAM. Mouse SLAM/CD150 cannot function as a receptor for either MeV or CDV, and attachment appears to be dictated by the amino acid sequence in the V loop of this protein (Hsu et al., 2001; Tatsuo et al., 2001). The V loop of nectin-4 is also involved in virus attachment but there are only 3 amino acids that differ in the V domain of the dog homologue, and 6 different amino acids in the V domain of mouse, when compared to the human protein sequence. Therefore, it may be possible to follow the release of MeV and CDV particles in a human/dog SLAM transgenic mouse having a background where interferon production or function is limited (Ferreira et al., 2010; Ohno et al., 2007; Welstead et al., 2005).

Overexpression of dog nectin-4 in interferon-deficient Vero cells facilitates CDV entry, productive infection, and syncytium formation. However, we also observed low levels of CDV production without syncytia formation in Vero cells lacking dog nectin-4. This was previously observed with MeV infections of Vero cells (Hashimoto et al., 2002). These single-infected cells have been reported in MeV-infected CHO, COS-1, and Vero cells at a frequency of 2–3 logs below that of SLAM-infected. RNAi treatment or polyclonal antibodies directed against PVRL4 did not affect these single cell background infections. Vero cells have depressed innate immunity (Chew et al., 2009; Mosca and Pitha, 1986) and CDV has also been reported to attach to the low affinity heparan sulfate receptor in a non-specific manner (Fujita et al., 2007). We postulate that the virus can enter the cell by an F protein-independent mechanism such as macropinocytosis. This route of entry may or may not be relevant during *in vivo* infections but probably accounts for the high background of CDV production in cells lacking known receptors.

Our data demonstrated that dog nectin-4 facilitates CDV entry and cell-to-cell spread in cultured Vero cells that express this receptor. Using a binding assay based upon virus-specific antibodies and FACS analysis, we previously showed that nectin-4/PVRL4 was a *bona fide* receptor used during attachment of MeV to the host cell (Noyce et al., 2011). This is undoubtedly the case for CDV. *Pratakpiriya* et al. recently reported that dog nectin-4 is an epithelial receptor for CDV, although the origin and sequence of dog nectin-4 was not mentioned (Pratakpiriya et al., 2012). RNAi was not used to verify receptor usage by knocking down nectin-4 expression. We also showed that dog nectin-4 functioned equally well or better than dogSLAM as a receptor and proved that it was involved in syncytia formation and cell-to-cell spread of CDV. The other group also suggested that nectin-4 functioned as a receptor in the brain using immunofluorescence microscopy, but this must be substantiated further. Further studies using primary epithelial cells from CDV susceptible species must also be performed to show how infected immune cells or virus access the nectin-4 receptor, which is normally localized to the adherens junctions between cells. As was previously done with MeV, we are testing the susceptibility of dog adenocarcinomas to CDV as a possible prelude to oncolytic therapy against various cancers.

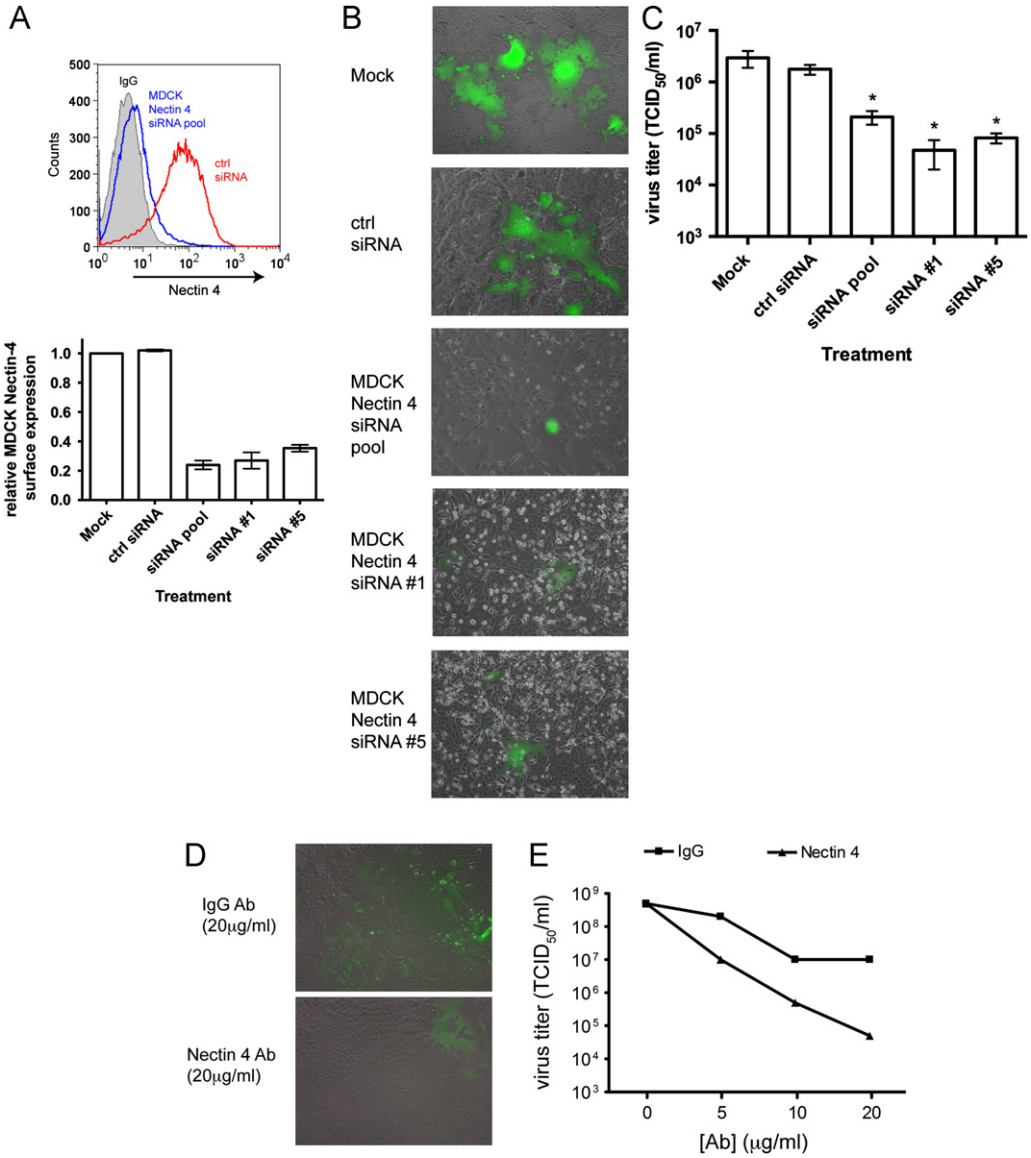

Fig. 6. Wild-type CDV 5804PeH requires dog nectin-4 to efficiently infect cells. Vero.MDCKNectin-4 cells were transfected with a scrambled oligonucleotide control (ctrl siRNA), a pool of siRNAs specific for MDCK nectin-4 (siRNA pool), or individual siRNAs specific for MDCK nectin-4 (siRNA #1 or siRNA #5). (A) Nectin-4 surface expression was detected with a PE-conjugated nectin-4 antibody following gene knockdown with control siRNA (red line) or MDCK nectin-4 siRNA (blue line) (upper panel). A graphical representation of the relative surface expression of MDCK Nectin-4 following siRNA treatment in three independent experiments is shown in the bottom panel. Data are presented as means ± SEM. (B) Nectin-4 RNAi results in a decrease in CDV infection and syncytia formation compared to Mock or control siRNA treatment. (C) Titration of CDV 72 h post infection in siRNA-treated Vero.MDCK nectin-4 expressing cells. At three days post infection, cells were harvested and virus titrations were performed on VerodogSLAMtag cells. Data are presented as means ± SEM from three independent experiments. Statistically significant differences (ANOVA, $P < 0.05$) are indicated by astericks. (D) Human nectin-4 antibodies inhibit wild-type CDV infection in Vero cells expressing MDCK nectin-4. Vero.MDCKNectin-4 cells were grown on glass coverslips and incubated with varying concentrations of goat IgG or goat anti-human nectin-4 for 30 min prior to, and during a 1 h adsorption of wild-type CD5804PeH. Virus infection and syncytia formation following CDV infection was monitored by fluorescence microscopy 3 days post infection. (E) Virus-infected Vero.MDCKNectin-4 cells treated with polyclonal antibodies were harvested 3 days post infection and titered on VerodogSLAMtag cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Materials and methods

Cell culture and virus infections

Vero cells, MDCK-super tubes (ATCC CRL-2285) and MDCK-super domes (ATCC CRL 2286) were purchased from the American Type Culture Collection (Manassas, VA). MDCK (ATCC CCL-34) cells and Phoenix cells were obtained from Dr. Craig McCormick and MDA-MB-231 and MCF-7 human breast cancer cell lines were generously provided by Dr. David Hoskin (Dalhousie, University). The recombinant Ichinoise-B 323 (IC323) wild type measles virus (MeV) isolate expressing the enhanced green fluorescent protein (eGFP) reporter gene (IC323-EGFP wtMeV) was obtained from Dr. Roberto Cattaneo (Mayo Clinic, Rochester). The recombinant wild-type CDV 5804PeH strain expressing eGFP (von Messling et al., 2004) was provided by Dr. Veronika von Messling (Duke-NUS, Singapore). Wild type MeV and CDV were propagated in Vero human SLAM and VerodogSLAMtag cells, respectively.

Molecular cloning of dog nectin-4 cDNA and V domain mutant

Total RNA was extracted from either Labrador retriever (LR) placenta (generously provided by Mr. Don Bates of Doindogs kennel, Dartmouth, NS), papillon placenta (Ms. Stephanie Callahan-Corkum and Ms. Mary Taplin of Country Critter Sitters,

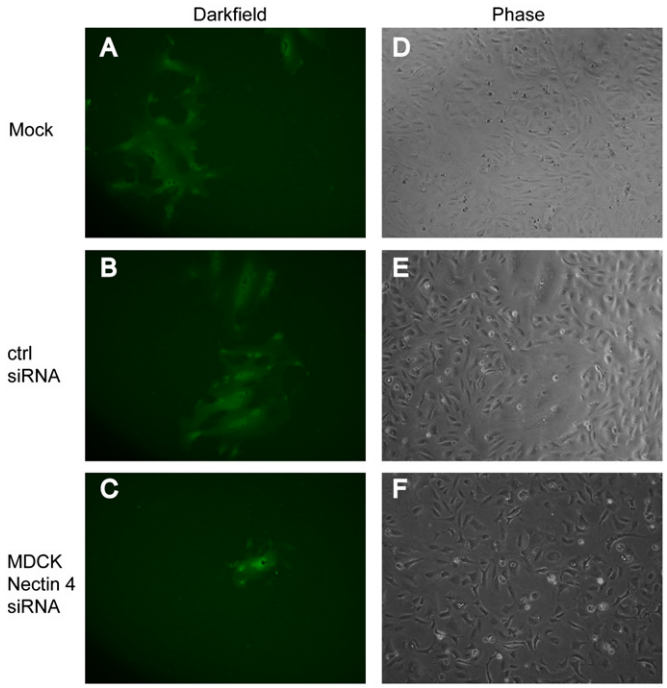

Fig. 7. Dog nectin-4 is required for cell to cell spread of wild-type CDV 5804PeH. Vero.MDCKNectin-4 cells were treated with scrambled oligonucleotides (ctrl siRNA) or a pool of MDCK nectin-4 siRNAs. After 72 h these cells were mixed with VerodogSLAMtag cells previously infected with CDV 5804PeH at an MOI of 4 for 90 min (ratio 5:1, VeroMDCK nectin-4:infected VerodogSLAMtag cells). The cells were subsequently overlaid with methylcellulose, and infectious foci were visualized by fluorescence microscopy 3 days following cell mixing.

Seaforth, NS), or MDCK cells and two micrograms was reverse transcribed with SuperScript III (Life Technologies) using a random hexamer primer. To amplify the cDNA encoding dog nectin-4, we performed PCR using consensus primers derived from the 5′ and 3′ regions of human, bovine, and mouse nectin-4; sense (5′-CCAATGCCTCTATCCCTGGGAGCCGAG-3′) and antisense (5′-GGAGGCAGGCCTGGGTCAGACCAGGTG-3′). Nested PCR was subsequently performed using sense and antisense primers containing *Nhe*I and *Eco*RI restriction sites, respectively, at the 5′ ends. The purified PCR product from either LR placenta or MDCK cDNA was ligated into pcDNA 3.1 (−) (Life Technologies) and subsequently sequenced. Pairwise protein alignments were performed using Geneious (Drumond et al., 2010) sequence alignment software. The individual immunoglobulin-like domains of nectin-4 were mapped using the ScanProsite tool (de Castro et al., 2006).

Creation of stable cell lines by retrovirus transduction

To create stable cell lines expressing dog nectin-4 from either LR placenta or MDCK cells, dog nectin-4 was sub cloned into the pBMN-IRES-Neo retrovirus (a kind gift from C. McCormick (Dalhousie University) using the *Bam*HI and *Eco*RI restriction sites. Human nectin-4 was subcloned into the pBMN-IRES-Neo retrovirus using the BamHI and *Eco*RI restriction sites. Phoenix cells were subsequently transfected using Lipofectamine 2000 and supernatant containing MDCK nectin-4- or LR nectin-4-expressing viruses were collected at 3d post transfection. Supernatants were briefly clarified at $1000 \times g$ for 5 min and subsequently incubated with Vero or MDA-MB-231 cells in the presence of 12 μg/ml polybrene for ninety minutes at $1000 \times g$. Forty-eight hours post retrovirus transduction, the Cells were sub-cultured in the presence of either 1000 μg/ml G418 for 14 days or until no cells were present on the control cell plate. These cells were tested for nectin-4 expression by flow cytometry and indirect immunofluorescence microscopy before being used for virus infections.

Virus infections

MDA-MB-231, MCF-7, and MDCK cell lines were seeded into 12-well plates so they were semi-confluent approximately 24 h later. Cells were infected with 5804PeH wtCDV at an MOI of 5.0 $TCID_{50}$/cell for 1 h at 37 °C. Cell monolayers were washed twice with PBS and incubated with DMEM containing 5% FCS. Images of infected monolayers were captured 4 days post infection and overlaid with phase micrographs.

Vero cells stably expressing empty vector (Vero.Neo), human nectin-4 (Vero.hNectin-4), MDCK nectin-4 (Vero.MDCKNectin-4), or Labrador retriever placental nectin-4 (Vero.LRNectin-4) were seeded into 12-well plates so they were confluent approximately 24 h later. Vero cells stably expressing human (Vero.hSLAM) or dog (Vero.dogSLAMtag) SLAM were used as positive controls for wtMeV or wtCDV infection and replication, respectively. Cells were infected with either IC323 EGFP wtMeV or 5804PeH wtCDV at an MOI of 0.05 or 0.5 $TCID_{50}$/cell, respectively, for 1 h at 37 °C. Cell monolayers were washed twice with PBS and replaced with DMEM containing 5% FCS. Cells were harvested at day 1, 2, and 3 for wtCDV infections and at 32 h for wtMeV infections. $TCID_{50}$ titers were determined by 50% end-point titration on Vero.dogSLAMtag or Vero.hSLAM cells for wtCDV and wtMeV, respectively, according to the Spearman-Kärber method. Data were expressed as means ± the standard error of the mean.

Virus spread assay

To assess the impact of nectin-4 on spread of CDV to non-infected cells expressing dog nectin-4, Vero.dogSLAMtag cells were infected at an MOI of 4. After 90 min, the virus inoculum was removed and cells were washed, trypsinized, and mixed at a 1:5 ratio with Vero.MDCKNectin-4 cells that had previously been transfected for 72 h with 50 pmol of control siRNA or a pool siRNA specific to knockdown MDCK nectin-4 mRNA. Three hours after seeding, the medium was removed and cells were overlaid with DMEM supplemented with 5% FCS and 1.5% methylcellulose (Sigma). Infectious foci were imaged 72 h after mixing.

Flow cytometry

To determine the cell surface expression of dog nectin-4 on Vero stable cell lines, cells were analyzed by flow cytometry. Briefly, cells were washed with PBS, non-enzymatically dissociated and subsequently blocked in 2.5 μg of normal human IgG (R&D Systems) for 10 min on ice followed by the addition of 10 μl of either PE-conjugated human nectin-4 (R&D Systems FAB2659P) or PE-conjugated mouse IgG2B isotype control (R&D Systems IC0041P) antibodies for 45 min on ice. Cells were washed twice in PBS containing 1% BSA, 5 mM EDTA, and 0.1% sodium azide and then fixed in 1% paraformaldehyde. Samples were run on a Cyan ADP Flow Cytometer (Beckman Coulter) and data were processed using FlowJo cytometry analysis software (Tree Star, Inc).

Confocal microscopy

Vero nectin-4 stable cell lines grown on coverslips were fixed in 4% paraformaldehyde (10 min) and blocked with 5% FCS in PBS for 1 h at room temperature. Nectin-4 was detected by incubating the cells with goat anti-human nectin-4 (R&D Systems AF2659) at

5 μg/ml in PBS containing 5% FCS for 45 min at room temperature. Cells were subsequently stained with fluorophore-conjugated secondary antibodies for 30 min at room temperature. Nuclear DNA was stained (20 min) with TO-PRO-3 stain (Life Technologies) in PBS containing 0.1% Triton X-100. Cells were mounted with fluorescent mounting medium and images were acquired with ZEN 2008 imaging software on a Zeiss LSM 510 upright laser scanning confocal microscope. Z-sections were captured with a 63 × Plan APOCHROMAT (1.4NA) objective lens at 0.3 μm increments and processed using ZEN 2011 lite and Adobe Photoshop CS3 using only linear adjustments.

siRNA inhibition of MDCK nectin-4

siRNA specific to MDCK nectin-4 and negative-control oligonucleotides (D-001810-10–05) were designed and synthesized by Dharmacon (Lafayette, CO). A pool of four oligonucleotide sequences targeting MDCK nectin-4 were used: MDCK nectin-4 siRNA #1 (sense, 5′-GGG CAG AGG ACG AGG AAG AUU-3′), MDCK nectin-4 siRNA #3 (sense, 5′-AGC AGA UGA CCC AGA AAU AUU-3′), MDCK nectin-4 siRNA #5 (sense, 5′-UCA AAC AGG CCA UGA ACC AUU-3′), and MDCK nectin-4 siRNA #7 (sense, 5′-CUC CAG GAC CAG AGG AUC AUU-3′). Briefly, a total of 50 pmol of either the non-targeting control siRNA, the MDCK nectin-4 pool (12.5 pmol of each siRNA), or individual MDCK nectin-4 siRNAs (50 pmol of siRNA ♯1 or ♯5) were reverse transfected into Vero-MDCK nectin-4-expressing cells with lipofectamine 2000 (Life Technologies, Burlington ON) according to the manufacturer's instructions. Transfected cells were allowed to grow for an additional 48 h before being reverse transfected with siRNA duplexes again. Three days following the initial transfection, cells were infected with 5804 PeH CDV at an MOI of 0.1. Infected cells were viewed by fluorescence and phase microscopy using a Leica DMI4000B inverted microscope (Leica Microsystems) 3d post infection and then harvested to determine virus titers.

Data analysis

Data were expressed as means ± the standard error of the mean unless otherwise indicated. Analysis of variance (ANOVA) was performed to identify statistically significant differences in CDV 5804PeH titrations following nectin-4 knockdown. *P* values below 0.05 were considered statistically significant. Overlays of phase and fluorescence micrographs were processed using only linear adjustments in Photoshop CS3.

Nucleotide sequence accession numbers

The GenBank accession numbers for Labrador retriever nectin-4 and MDCK cell nectin-4 cDNA sequences are JX629803 and JX629804, respectively. GenBank accession numbers for papillon nectin-4 variants 1 and 2 are KC165685 and KC165686.

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